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siRNA targeting Vaccinia virus double-stranded RNA binding protein [E3L] exerts potent antiviral effects

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Abstract

The Vaccinia virus gene, *E3L*, encodes a double-stranded RNA [dsRNA]-binding protein. We hypothesized that, owing to the critical nature of dsRNA in triggering host innate antiviral responses, *E3L*-specific small-interfering RNAs [siRNAs] should be effective antiviral agents against pox viruses, for which Vaccinia virus is an appropriate surrogate. In this study, we have utilized two human cell types, namely, HeLa and 293T, one which responds to interferon [IFN]-β and the other produces and responds to IFN-β, respectively. The antiviral effects were equally robust in HeLa and 293T cells. However, in the case of 293T cells, several distinct features were observed, when IFN-β is activated in these cells. Vaccinia virus replication was inhibited by 97% and 98% as compared to control infection in HeLa and 293T cells transfected with *E3L*-specific siRNAs, respectively. These studies demonstrate the utility of *E3L*-specific siRNAs as potent antiviral agents for small pox and related pox viruses.

Keywords: RNAi; siRNA; Vaccinia; E3L; Innate immunity

Introduction

There has been a recent dramatic increase in interest in the study of Smallpox and related pox viruses using modern molecular virology tools. Recent incidences of monkey pox virus infection of humans in the USA suggest its potential as an emergent epidemiological agent. As well, the current geopolitical scenario has implicated the use of Smallpox virus as a potential bio-weapon. Smallpox was eradicated before the emergence of recombinant DNA technologies and has therefore not been fully studied with contemporary methods. Smallpox pathogenesis is not understood adequately enough to suggest detailed molecular mechanisms which are responsible for the high death rate associated with the disease (Esposito and Fenner, 2000). Vaccinations against Smallpox are complicated, and substantial morbidity is associated with existing vaccines (Moss, 2001). This is even further complicated by the presence of a significant immunocompromised human population. Exploring the role of siRNAs as effective antiviral agents against pox viruses therefore merits consideration.

Vaccinia virus is an appropriate surrogate for studying Smallpox viral pathogenesis. It is a relatively safe virus as it does not cause serious disease in most immunocompetent humans or animals (Diven, 2000). The genomes of both viruses share substantial similarity, and many of the critical genes are strongly conserved in the two viruses. For example, *E3L*, encoding a dsRNA binding protein possesses an identical DNA sequence in the Vaccinia and Variola genomes.

Immediately after viral infection, critical host defenses, comprising the innate immune response, are induced. Innate immune response makes a critical contribution to the activation of adaptive immunity. However, there is a delay of 4 to 7 days before the initial adaptive immune response takes place. Hence, the innate immune response plays a critical role in initial viral

Abbreviations: IFN, Interferon; RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; β-gal, β-galactosidase; PKR, dsRNA-dependent Protein Kinase C.

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clearance (Janeway et al., 2001). Vaccinia virus attempts to subvert the innate immune response by secreting proteins that inactivate complement (Kotwal et al., 1990), inhibit the IFN response (Beattie et al., 1995; Carroll et al., 1993; Colamonici et al., 1995; Symons et al., 1995), buffer against the inflammatory response (Alcami et al., 1998) and prevent activation of natural killer cells (Alcami and Smith, 1995). These proteins are likely responsible for potent and often lethal infections associated with the Smallpox virus (Diven, 2000). The proteins involved in inhibiting the IFN response and preventing the activation of natural killer cells include E3L (Chang et al., 1992), B18R (Colamonici et al., 1995), K3L (Carroll et al., 1993) and B8R (Alcami and Smith, 1995).

The Vaccinia *E3L* gene encodes a 25 kDa polypeptide synthesized early during the viral life-cycle (Chang et al., 1992). E3L is a dsRNA binding protein encoded by Vaccinia virus to bypass the effects of IFNs. E3L is also anti-apoptotic and oncogenic (Garcia et al., 2002). The critical role of E3L has been demonstrated in several studies (Brandt et al., 2005; Langland and Jacobs, 2002, 2004; Liu et al., 2001; Ludwig et al., 2005; Xiang et al., 2002). *B18R* encodes a soluble IFN-α/β receptor (Colamonici et al., 1995). B18R has sequence similarities with type I IFN receptors and thus binds IFN-α/β with high affinity. However, B18R is less species-specific than the type I IFN receptors, enabling it to bind type I IFNs of several species (Colamonici et al., 1995; Symons et al., 1995). *K3L* encodes a homolog of the eukaryotic translation initiation factor eIF-2α. K3L competitively inhibits phosphorylation of eIF-2α (Carroll et al., 1993; Davies et al., 1993). *B8R* encodes a soluble IFN-γ receptor that binds and antagonizes IFN-γ (Alcami and Smith, 1995). Thus, it appears that *E3L* is the critical gene that initiates the cascade that diminishes the effectiveness of the innate immune response (Fig. 1).

The ability of E3L to function as a dsRNA binding protein ensures that dsRNA is sequestered and RNAi is suppressed (Li et al., 2004). dsRNA induces a multifaceted response in higher eukaryotes, even when a single molecule is present (Cullen, 2002; Marcus and Sekellick, 1977). DNA viruses, such as Vaccinia, generate dsRNA as a result of overlapping convergent transcription. Late transcripts of Vaccinia are long and heterogeneous. The early termination signal, TTTTTNT, is not recognized by the late transcription system. Terminal heterogeneity combined with transcription from both DNA strands explains the ability of late transcripts to self-anneal or anneal with early transcripts to form ribonuclease resistant hybrids in vitro (Moss, 1996). dsRNA is an important pathogen-associated molecular pattern that might provide enhanced stimulation of innate immunity (Whitmore et al., 2004). dsRNA binding proteins, encoded by several non-vertebrate RNA viruses and vertebrate viruses, play a role in suppressing RNAi (Dasgupta et al., 1998; Denzler and Jacobs, 1994; Kaufman, 1999; Lichner et al., 2003). In mammalian cells, RNAi functions independently of the IFN-induced pathways (Brummelkamp et al., 2002; Cullen, 2002; Dave and Pomerantz, 2003; Sui et al., 2002; Yu et al., 2002). Thus, an siRNA-based approach targeting *E3L* is likely to inhibit the virus by triggering RNAi, as well as lead to an enhancement of the innate immune response. Furthermore, an siRNA-based strategy that enables destruction of the entire transcript will probably prevent any of the diverse functions of *E3L* to be executed by its discrete domains [such as nuclear localization, Z-DNA-binding, dsRNA-dependent Protein Kinase C [PKR] binding and dsRNA binding] (Brandt and Jacobs, 2001). Simultaneously targeting *E3L* and *B18R* could possibly lead to a cumulative enhancement of the antiviral effects by allowing the antiviral effects of IFNs to be manifested. In the present study, we have utilized *E3L* and *B18R* as targets for siRNAs in two cell types, HeLa and 293T. HeLa cells were utilized for this study because *E3L* deletions render the virus incapable of replication in this cell type (Chang et al., 1995). As well, 293T cells were utilized since they are able to both produce and respond to IFNs, while HeLa cells are able to respond but not produce IFNs. The present study enables us to determine whether these siRNAs can be used as antiviral tools.

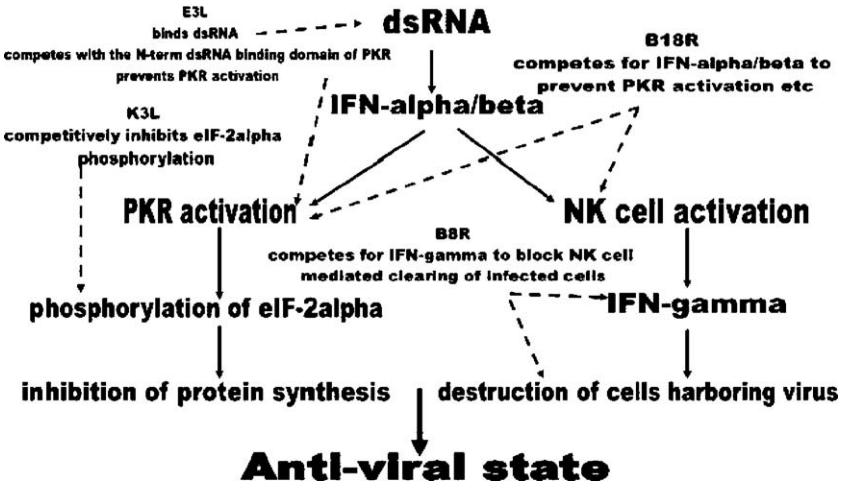

Fig. 1. Molecular pathways by which Vaccinia subverts host defense mechanisms. Vaccinia virus masks the well-known pathogen-associated molecular pattern, dsRNA, by producing the dsRNA binding protein E3L. Other key viral genes that are believed to play a role in preventing the generation of antiviral state and an innate immune response are *B18R*, *B8R* and *K3L* (see text for details and references).

Results

E3L-specific siRNAs inhibit Vaccinia virus replication and production of progeny virions

To test whether the *E3L*- and *B18R*-specific siRNAs exert antiviral effects, HeLa cells were transfected with these moieties. The transfected cells were then infected with Vaccinia virus at MOIs of 0.1 or 1.0 pfu/cell. The antiviral effect was assayed at 2 dpi since it is known from our previous results that, in similar systems, maximal antiviral effect is exerted at 2 dpi (Dave and Pomerantz, 2004). The inhibition of viral replication was assessed by measuring the β-gal enzyme activity in cell extracts at 2 dpi. A plaque assay was used to assess the inhibition of progeny virion production at 2 dpi. The plaque assay measures the levels of intracellular mature virions.

At an MOI = 0.1 pfu/cell, *E3L*-specific siRNAs inhibited viral replication to below detection levels [data not shown]. However, at an MOI = 1.0 pfu/cell, the *E3L*-specific siRNAs inhibited viral replication to 7.6% of control infection [P = 0.009] (Fig. 2). At neither of these MOIs were the *B18R*-specific siRNAs able to inhibit viral replication [P = 0.314 at MOI = 0.1 pfu/cell; P = 0.966 at MOI = 1.0 pfu/cell]. When *E3L*- and *B18R*-specific siRNAs were transfected simultaneously, viral replication was inhibited to below detection levels and 4.9% of control infection at MOI = 0.1 pfu/cell and 1.0 pfu/cell respectively. The antiviral effect of *E3L*-specific siRNAs was reflected similarly on the production of infectious progeny virions. At an MOI = 1.0 pfu/cell, the *E3L*-specific siRNAs inhibited the production of progeny virions to 4.5% of control infection [P = 0.009] (Fig. 3). We therefore concluded that *E3L*-specific siRNAs have the ability to potently inhibit Vaccinia virus replication and production of progeny virions in HeLa cells. *B18R*-specific siRNAs failed to do so.

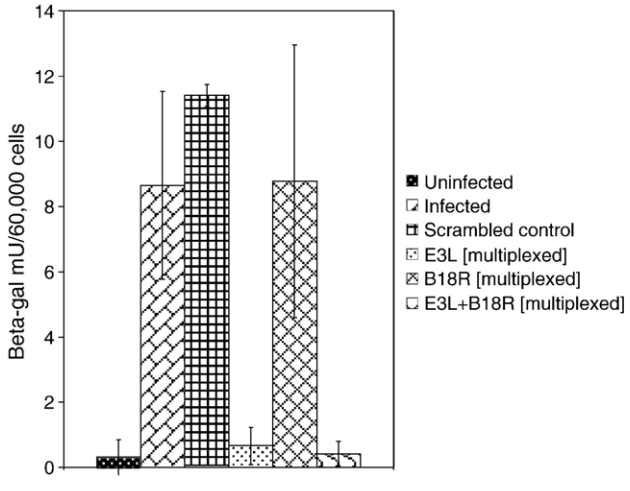

Fig. 2. *E3L*-specific siRNAs inhibit viral replication in HeLa cells infected with Vaccinia virus. HeLa cells were transfected with *E3L*- and *B18R*-specific siRNAs, along with scrambled control siRNAs. siRNAs were multiplexed, and four different siRNAs were used for each gene. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. β-gal activity was measured in cell extracts at 2 dpi. The means of three experiments and standard deviations are indicated.

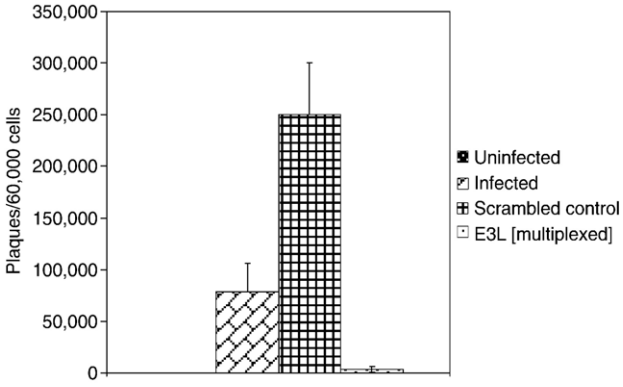

Fig. 3. *E3L*-specific siRNAs inhibit virion production in HeLa cells infected with Vaccinia virus. HeLa cells were transfected with *E3L*-specific siRNAs, along with scrambled control siRNAs. siRNAs were multiplexed, and four different siRNAs were used for *E3L*. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. The intracellular mature virion concentration within HeLa cells was estimated by plaque titration on B-S-C-1 cells at 2 dpi. The means of three experiments and standard deviations are indicated.

As *E3L*-specific siRNAs had a consistent antiviral effect, we therefore decided to individually evaluate the antiviral effects of each of the four *E3L*-specific siRNAs that were multiplexed in previous experiments. HeLa cells were transfected with individual siRNAs, along with a scrambled control, and at the end of transfection, infected with Vaccinia virus at an MOI = 1.0 pfu/cell. β-gal activity was measured in cell extracts at 2 dpi (Fig. 4). E3L-C siRNA exerted the strongest and consistently robust antiviral effect, inhibiting viral replication to 3.5% of control infection [P = 0.003]. E3L-A and E3L-B siRNAs inhibited viral replication to 17.2% and 64.3% of control, respectively [P = 0.000003 and 0.008]. E3L-D siRNA failed to inhibit viral replication. Since E3L-C siRNA was the most potent of the four *E3L*-specific siRNAs tested, it was used in all subsequent experiments to target *E3L*.

To evaluate the efficacy of E3L-C siRNA, the siRNAs were transfected into HeLa cells and at the end of transfection infected with Vaccinia virus at different MOIs ranging from 0.1 to 4.0 pfu/cell. E3L-C siRNAs inhibited viral replication in the range of 14.6 to 23.85% of control infection (Fig. 5). Maximum inhibitory effect was observed at MOIs ranging from 1.0 to 4.0 pfu/cell. At MOIs greater than 2 pfu/cell, HeLa cells that were not transfected with E3L-C siRNAs exhibited cytopathic effects. Increasing MOI from 2 pfu/cell to 4 pfu/cell did not proportionately increase viral replication in control infections. These results suggest that E3L-C siRNA is an efficient antiviral moiety and has substantial efficacy, even at very high MOIs that otherwise generate cytopathic effects in infected HeLa cells.

Having established the antiviral nature of *E3L*-specific siRNAs in HeLa cells, we next tested them in 293T cells. Cells were transfected with E3L-C siRNA and *B18R*-specific siRNAs and at the end of transfection infected with Vaccinia virus [MOI = 1.0 pfu/cell]. β-gal activity was measured in cell extracts at 2 dpi (Fig. 6). Cells transfected with E3L-C siRNAs exhibited a strong anti-proliferative effect, a likely consequence of IFN-β activation [data not shown]. To ensure that infected

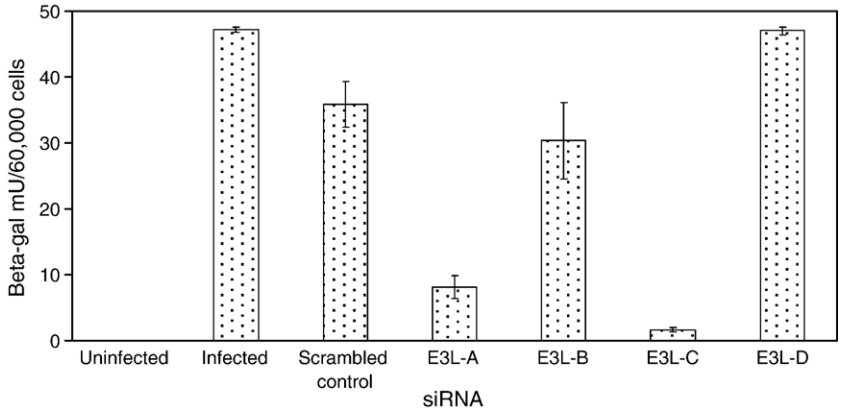

Fig. 4. E3L-C siRNAs were most efficient in inhibiting viral replication in HeLa cells infected with Vaccinia virus. HeLa cells were transfected with *E3L*-specific siRNAs along with scrambled control siRNAs. Antiviral efficacy of each of the four *E3L*-specific siRNAs was determined. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. β-gal activity was measured in cell extracts at 2 dpi. The means of three experiments and standard deviations are indicated.

cells had not undergone lysis and that β-gal activity was not being measured in uninfected cells, we assayed both the cell extracts as well as culture supernatants. No β-gal activity was detected in any of the culture supernatants. As in HeLa cells, *B18R*-specific siRNAs failed to exert an antiviral effect in 293T cells. E3L-C siRNA inhibited viral replication to 2.54% [P = 0.0004] and 2.84% [P = 0.0004] of control infection, alone or in combination with *B18R*-specific siRNAs respectively. Thus, E3L-C siRNAs exert a potent antiviral effect in HeLa and 293T cells.

E3L-specific siRNAs down-regulate E3L and B18R transcripts

We next performed semi-quantitative analyses to verify changes in transcript levels of key viral genes, *E3L*, *B18R*, *K3L* and *B8R*. Messenger RNA levels were measured in total RNA samples isolated from HeLa and 293T cells transfected with E3L-C siRNAs and *B18R*-specific siRNAs and at the end of transfection infected with Vaccinia virus [MOI = 1.0 pfu/cell]. Total RNA was extracted at 2 dpi. E3L-C siRNAs were able to down-regulate *E3L* transcripts in HeLa and 293T cells (Fig. 7). A reduction in *E3L* transcripts paralleled a reduction in *B18R* transcripts. *B18R*-specific siRNAs did not seem to influence *B18R* transcripts nor did they exert any antiviral effect in either cell type tested. However, E3L-C siRNA possessed the ability to down-regulate *E3L* and *B18R* transcripts. Of note, E3L-C siRNA do not possess any sequence homology to *B18R* sequence.

Antiviral state generated by E3L-specific siRNAs involves distinct features in HeLa and 293T cells

K3L and *B8R* appear to be differentially regulated in HeLa and 293T cells transfected with E3L-specific siRNAs. In

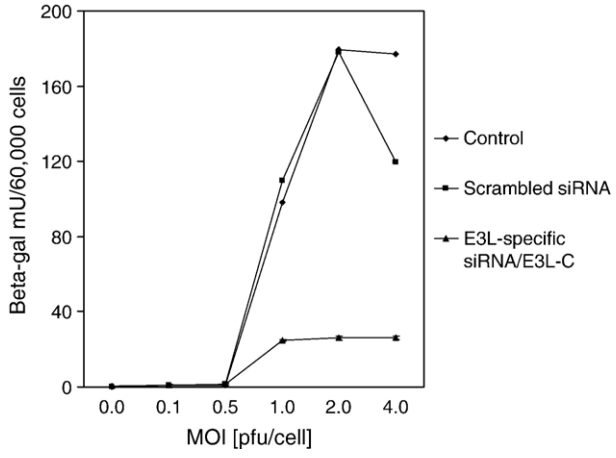

Fig. 5. E3L-C siRNAs efficaciously inhibit viral replication at varying levels of input virus in HeLa cells infected with Vaccinia virus. HeLa cells were transfected with E3L-C siRNAs and then infected at varying MOIs with the Vaccinia virus. β-gal activity was measured in cell extracts at 2 dpi. Control represents HeLa cells infected with the virus alone. The means of three experiments and standard deviations are indicated for E3L-C siRNA. For control and scrambled siRNA, the experiment was performed with one replicate.

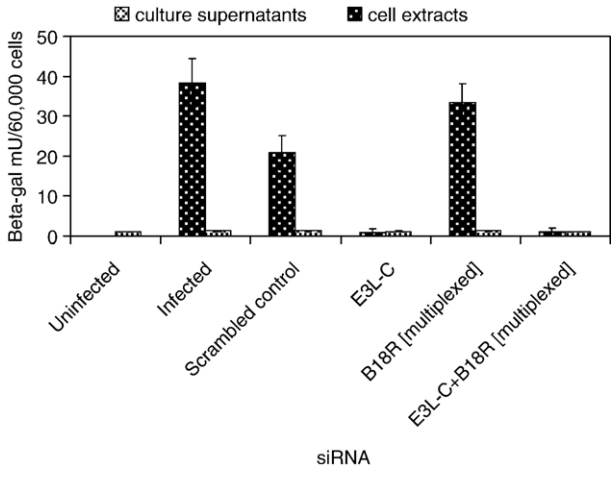

Fig. 6. E3L-C siRNAs inhibit viral replication in 293T cells infected with Vaccinia virus. 293T cells were transfected with E3L-C and *B18R*-specific siRNAs along with scrambled control siRNAs. E3L-C siRNA was used for *E3L*. Four different siRNAs were used for *B18R*. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. β-gal activity was measured in cell extracts and culture supernatants at 2 dpi. The means of three experiments and the standard deviations are indicated.

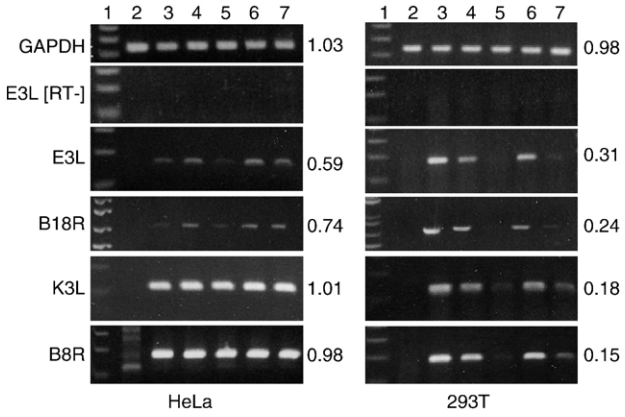

Fig. 7. Vaccinia virus genes involved in subverting the host defense mechanism behave differently in HeLa and 293T cells. HeLa and 293T cells were transfected with *E3L*- and *B18R*-specific siRNAs along with scrambled control siRNAs. The siRNAs were multiplexed, and four different siRNA were used for *B18R*. E3L-C siRNA was utilized for *E3L*. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. Total RNA was isolated at 2 dpi. Lane 1: 1 kbp ladder [Invitrogen], lane 2: uninfected HeLa or 293T cells, lane 3: cells infected with Vaccinia virus, lane 4: cells infected with Vaccinia virus and transfected with scrambled control siRNAs, lane 5: Cells infected with Vaccinia virus and transfected with E3L-C siRNAs, lane 6: cells infected with Vaccinia virus and transfected with *B18R*-specific siRNAs, lane 7: cells infected with Vaccinia virus and transfected with E3L-C siRNAs along with *B18R*-specific siRNAs. The numbers on the right side of each panel are the density ratio of the RT-PCR products with reference to the sample from cells infected with Vaccinia virus [lane 5 versus lane 3].

HeLa cells, transcript levels of neither of these genes were influenced by transcriptional down-regulation of *E3L*. However, in 293T cells, both gene transcripts were depressed when *E3L* transcripts were down-regulated. Transcriptional down-regulation of *K3L* and *B8R*, along with that of *E3L* and *B18R*, occurred only in 293T cells by E3L-C siRNA (Fig. 7).

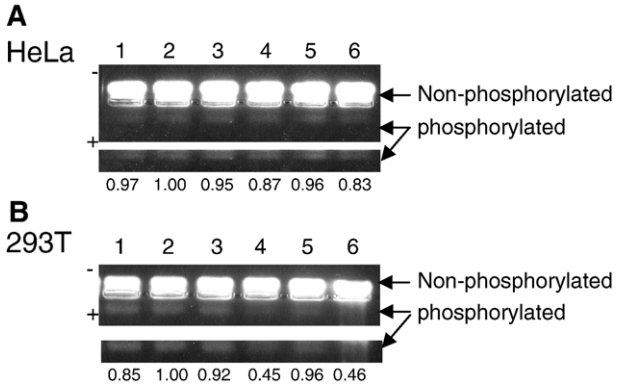

Fig. 8. *E3L*-specific siRNAs lead to greater inhibition of PKR activation in 293T cells as compared to HeLa cells. HeLa and 293T cells were transfected with *E3L*- and *B18R*-specific siRNAs along with scrambled control siRNAs. The siRNAs were multiplexed, and four different siRNA were used for *B18R*. E3L-C siRNA was used for *E3L*. Cells were infected at the end of transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. PKR activity was measured in cell extracts from HeLa cells [A] and 293T cells [B]. Lane 1: uninfected HeLa or 293T cells, lane 2: cells infected with Vaccinia virus, lane 3: cells infected with Vaccinia virus and transfected with scrambled control siRNAs, lane 4: cells infected with Vaccinia virus and transfected with E3L-C siRNAs, lane 5: cells infected with Vaccinia virus and transfected with *B18R*-specific siRNAs, lane 6: cells infected with Vaccinia virus and transfected with E3L-C siRNAs along with *B18R*-specific siRNAs. The numbers under each panel are the density ratio of the fluorescent phosphorylated peptide bands with reference to the sample from cells infected with Vaccinia virus.

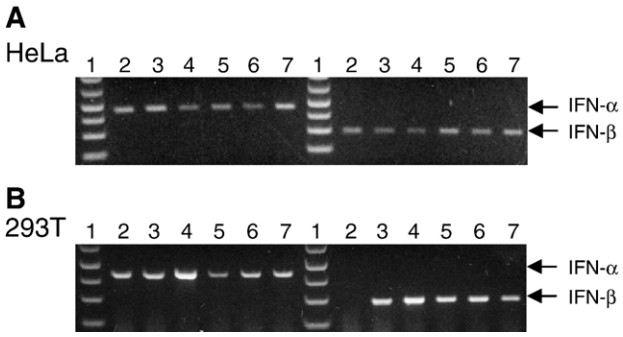

Fig. 9. IFN-β transcription is induced as a result of infection with Vaccinia virus in 293T cells. HeLa [A] and 293T [B] cells were transfected with *E3L*- and *B18R*-specific siRNAs, along with scrambled control siRNAs. The siRNAs were multiplexed, four different siRNA were used for *B18R*. E3L-C siRNA was used for *E3L*. Cells were infected after transfection with Vaccinia virus at an MOI of 1.0 pfu/cell. Total RNA was isolated at 2 dpi. Both panels show two sets of lanes 1 to 7. In set 1, IFN-α transcripts were detected, and IFN-β transcripts were detected in set 2. Lane 1: 1 kbp ladder [Invitrogen], lane 2: uninfected HeLa or 293T cells, lane 3: cells infected with Vaccinia virus, lane 4: cells infected with Vaccinia virus and transfected with scrambled control siRNAs, lane 5: cells infected with Vaccinia virus and transfected with E3L-C siRNAs, lane 6: cells infected with Vaccinia virus and transfected with *B18R*-specific siRNAs, lane 7: cells infected with Vaccinia virus and transfected with E3L-C siRNAs along with *B18R*-specific siRNAs.

To gain further initial insights into the molecular mechanism [s] that might be associated with the emergence of antiviral state upon transfection with E3L-C siRNAs, we investigated PKR activation and IFN-α/β expression at 2 dpi in HeLa and 293T cells infected with Vaccinia virus [MOI = 1.0 pfu/cell]. In HeLa cells, PKR was not activated post-infection, irrespective of the fact that cells were transfected with any siRNAs. There was slight PKR inhibition in HeLa cells transfected with E3L-C siRNAs. However, in 293T cells, PKR activation was inhibited to 45% in cells transfected with E3L-C siRNAs as compared to cells infected with Vaccinia virus only (Fig. 8). As well, in HeLa cells, IFN-α/β transcripts were constitutively expressed. However, in 293T cells, only IFN-α transcript expression was constitutive. IFN-β transcripts were expressed only in cells that were infected with the Vaccinia virus (Fig. 9). Hence, E3L-C siRNAs were able to generate an antiviral state in both cell types. However, inhibition of PKR activity and transcriptional down-regulation of *K3L* and *B8R* occurred only in 293T cells. The distinct features associated with generation of antiviral state in 293T cells might be attributed to the differential expression of IFN-β in this cell type.

Discussion

These data demonstrate that E3L-C siRNAs are effective in inhibiting Vaccinia virus infection. *E3L* encodes a dsRNA binding protein. Transcriptional down-regulation of this protein is likely to unmask the dsRNA. While HeLa cells are able to respond to IFN-β, they do not produce this moiety. On the other hand, 293T cells respond, as well as produce, IFN-β. That

antiviral effect of E3L-C siRNAs despite being equally robust in 293T cells possessed distinct features not observed in HeLa cells suggests that IFN-β plays a role in the generation of an antiviral state.

Down-regulation of *E3L* transcripts led to down-regulation of *B18R*, *K3L* and *B8R* transcripts. While cells transfected with E3L-C siRNA and infected with Vaccinia virus show down-regulation of *E3L* and *B18R* transcripts, however, that was not the case in cells transfected with *B18R*-specific siRNAs. The inability of *B18R*-specific siRNAs to inhibit viral replication is surprising. Since B18R binds to type I IFNs, the effect of targeting *E3L* and *B18R* should have been cumulative (Colamonici et al., 1995). The gene is perhaps not likely critical in controlling viral replication in the two cell types which were analyzed. No transcriptional down-regulation of *B18R* was observed. This could be because the gene is dispensable. Even when the siRNAs attempt to down-regulate the transcripts, the virus continues to grow and the transcript levels parallel viral replication. That all four of the *B18R*-specific siRNAs were ineffective is very unlikely, though it cannot be unequivocally ruled out. Although E3L binds dsRNA, it is unlikely that E3L binds to 21-bp siRNAs. In that case, the *E3L*-specific as well as *B18R*-specific siRNAs would not be able to exert antiviral effects. Clearly, that is not the case as *E3L*-specific siRNAs were effective. E3L-mediated inhibition of RNAi might be a possible explanation. We have observed that siRNA-mediated down-regulation of GAPDH is ineffective in the context of Vaccinia viral infection. In such a scenario, siRNA-mediated antiviral strategy targeting E3L might be the most effective approach. The ability of E3L-C siRNAs to target *B18R* and *E3L* transcripts suggests that it is an excellent antiviral moiety.

Unlike *B18R*, concomitant decrease of *K3L* and *B8R* transcripts appears to be dependent on the IFN producer phenotype of the cells. It is likely that IFN-β plays a pivotal role in enabling *E3L* to regulate transcription of both the genes. Several studies have demonstrated molecular interactions between the gene products of *E3L* and *K3L* (Davies et al., 1993; Langland and Jacobs, 2002). However, thus far, the ability of *E3L* to regulate transcription of *K3L* and *B8R* has not been demonstrated.

Our results demonstrate that response of PKR is dependent on the IFN producer phenotype of the cells. Similar observation was made in a study that utilized Δ*E3L* and Δ*K3L* Vaccinia viruses in HeLa and BHK cell types (Langland and Jacobs, 2002). Vaccinia virus carrying a deletion for *E3L* has been known to activate PKR in IFN-treated Cos-1 cells (Chang et al., 1992). However, we have not observed PKR activation in situations where E3L is possibly down-regulated [by *E3L*-specific siRNAs] in either cell type tested. Of note, *E3L*-specific siRNAs inhibited PKR in 293T cells. In HeLa cells, no significant changes in PKR activation were observed. Most of the studies with mutant viruses have treated cells with IFN (Chang et al., 1992; Langland and Jacobs, 2002). We have observed that, if 293T cells were treated with a substantial amount of poly[I][C] [100 μg/mL], a small quantity of IFN-β was detected by ELISA and PKR was activated [data not shown]. HeLa cells did not produce IFN-β, although transcripts for IFN-β were detected.

The inhibition of PKR as well as transcriptional down-regulation of *K3L* and *B8R* was observed only in 293T cells transfected with E3L-C siRNAs. While connection between *B8R* and PKR is unknown, a close relationship between *K3L* and PKR exists. *K3L* is a competitive inhibitor of and acts as a pseudo-substrate of PKR. As such, observations about the relationship between *K3L* and PKR are cell-dependent. In HeLa cells, *K3L* is not essential for viral replication, however, in BHK-1 cells, it plays a critical role paralleling *E3L* (Chang et al., 1992). The activation curve for dsRNA is bimodal, where low concentrations of dsRNA activate and high concentrations of dsRNA inhibit activation of PKR (Kaufman, 2002). The amount of dsRNA un-sequestered upon transcriptional down-regulation of *E3L* in the two cell types might be different and is the likely reason for distinct PKR activation profiles.

Taken together, our results suggest that siRNAs targeting *E3L* are effective in potently inhibiting Vaccinia virus. Thus far, siRNA-based antiviral approaches against Vaccinia virus and related poxviruses have not been described. The antiviral effects of *E3L*-specific siRNAs probably involved down-regulation of the protein as well. However, this has not been unequivocally established in this study. *E3L* very likely plays the initiating role in evading the initial innate immune response. As such, this approach has a strong potential of enhancing the innate immune response and thereby preventing a potent Vaccinia viral infection. Recently, two studies have described that presence of uridine-rich sequences, irrespective of the GC content, exerts a potent immunostimulatory effect through Toll receptors (Hornung et al., 2005; Judge et al., 2005). Neither motifs, 5′-UGUGU-3′ and 5′-GUCCUUCAA-3′, are present on the sense strand of any of the siRNA sequences that we have tested in the present studies. Thus, enhancement of innate immune response will be a direct consequence of the down-regulation of dsRNA binding protein E3L. The motif 5′-UGUGU-3′ occurs 149 times in the Vaccinia WR genome. The other uridine-rich sequence does not occur at all. As late stages of Vaccinia infection generates dsRNA, the 5′-UGUGU-3′ motif is likely to be incorporated. When dsRNA is un-sequestered, the resultant siRNA generated have the possibility of possessing the immunostimulatory motif, thereby further enhancing the innate immune response in a specific manner.

These studies demonstrate that, indeed, siRNA-based antivirals are potent and effective approaches against a poxvirus. As well, targeting the mechanisms by which poxviruses subvert the host's innate immune system will not only likely yield novel therapeutics but can functionally interrogate the complex viral: innate immune system interactions.

Materials and methods

Cells and viral stocks

HeLa, 293T and B-S-C-1 cells [ATCC] were maintained in Dulbecco's modified Eagle Medium containing 10% heat-

inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 50 U/mL penicillin G and 50 μg/mL streptomycin. Recombinant Vaccinia virus [VSC-56] expressing the β-galactosidase [β-gal] gene at the thymidine kinase locus was used in all experiments. β-gal expression was under the control of the natural p7.5 early/late promoter element (Bronte et al., 1997). Viral stocks were prepared in HeLa cells, as described previously (Earl and Moss, 1991). Viral concentrations were determined by plaque titration on B-S-C-1 cells.

siRNA selection, preparation and transfection

Web-based tools from Ambion, Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology and the National Center for Biotechnology Information websites were used for selection of siRNA sequences and for Blast searches. siRNA sequences were Blast-searched either in the EST-Human database or databases containing viral nucleic acid sequences. In either case, Blast searches were for "short nearly exact matches". Chemically synthesized siRNAs were utilized in all the experiments [Dharmacon]. The siRNA duplexes with 3′-AA overhangs were duplexed, desalted, purified and 2′-deprotected. siRNA duplexes [20 μM] were stored in 6 mM HEPES-KOH buffer, pH 7.5 containing 20 mM KCl and 0.2 mM $MgCl_2$ at −20 °C. Scrambled [negative control] siRNAs [Ambion] were used to ensure the specificity and statistical significance of the observed antiviral effects. Statistical significance of the antiviral effects was determined with reference to samples from cells infected with Vaccinia. However, during the course of the study, we determined the specificity and statistical significance of antiviral effects with reference to samples from cells infected with Vaccinia as well as cells transfected with Scrambled control siRNA and then infected with Vaccinia.

The siRNA sequences used to down-regulate *E3L* were E3L-A [5′-AAU ACU CUC CCG UCG AUG UCU-3′], E3L-B [5′-AAG ACU UAU GAU CCU CUC UCA-3′], E3L-C [5′-AAU AUC GUC GGA GCU GUA CAC-3′] and E3L-D [5′-AAC GCU CGU CAA UAU AGA UCU-3′]. The following four sequences were used to down-regulate *B18R*; B18R-A [5′-AAA GUG UCC UCC UAU UGA AGA-3′], B18R-B [5′-AAC CUG CCA AUA UAA CAU GCA-3′], B18R-C [5′-AAU CCA UCC GGA UGG CUU AUA-3′] and B18R-D [5′-AAA UGU CGU GGA CAC AAC UAU-3′]. siRNAs were transfected, as described previously (Dave and Pomerantz, 2004). For HeLa cells, siPort Lipid [Ambion] was used to transfect siRNA. Lipofectamine 2000 [Invitrogen] was used to transfect 293T cells. All transfections were in 6-well plates, 6×10^4 cells were plated in each well in DMEM containing 10% heat-inactivated FBS and 2 mM L-glutamine. Approximately 18 h after plating, when the cells had reached 30% confluence, siRNA [10 pmol/well] siPort lipid [Ambion] or Lipofectamine [Invitrogen] complexes in Optimem-I were added for transfection for 5 h. Lipid complexes were generated according to manufacturer's instructions. At the end of the 5-h transfection, cells were infected with the recombinant Vaccinia virus [MOI; 0.1 or 1.0 pg p24/cell] for 2 h. At the end of the 2-h period, cells were washed with phosphate-buffered saline [PBS] to remove input virus. Cells were cultured in Optimem-I medium without any supplements for 2 days. Viral replication was assessed by measuring β-gal activity in cell extracts, and production of progeny virions was assessed by measuring the quantity of intracellular mature virions at 2 days post-infection [dpi].

β-gal enzyme assay

The β-gal enzyme assay kit [Promega] was used to measure the activity of the enzyme in cell extracts from HeLa or 293T cells, essentially according to manufacturer's instructions with a few modifications. Adherent cells were harvested with trypsin. Cells were washed with chilled PBS containing 5% FBS followed by another wash with PBS. Cells [6×10^4] were finally extracted in 150 μL of 1× Reporter Lysis Buffer provided in this kit. The assay measures the ability of β-gal to hydrolyze o-nitrophenyl-β-D-galactopyranoside [colorless] to o-nitrophenyl [yellow]. The assay is expected to maintain a linear range with respect to standards, if it is performed for 30 min. That is not typically the case with test samples, and the reaction is terminated to ensure that the values can be interpolated from the standard curve. Hence, values are typically compared within a particular experimental set and not across different sets of experiments.

PKR activation assay

A PepTag® Non-Radioactive PKR assay kit [Promega] was used to determine PKR activation according to manufacturer's instructions with a few modifications. Cells were harvested as described above for the β-gal enzyme assay and extracted in 200 μL chilled buffer, 25 mM Tris–HCl, pH 7.4, containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% TritonX-100, 10 mM β-mercaptoethanol, 1 μg/mL Leupeptin, 1 μg/mL Aprotinin and 1 mM PMSF. 10% of the sample was used in the assay, and the entire reaction mix [50 μL] was analyzed by electrophoresis through a 0.8% agarose gel in 50 mM Tris–HCl, pH 8.0. The fluorescent peptides were visualized by UV illumination. The gel photographs were scanned and further analyzed for density comparison by using IQ Mac v1.2 software [Molecular Dynamics]. The density ratio of the fluorescent phosphorylated peptide bands in test samples was determined with reference to the sample from cells infected with Vaccinia virus.

Reverse transcriptase-polymerase chain reaction [RT-PCR]

Total RNA was extracted from HeLa and 293T cells [transfected with siRNA and infected with Vaccinia] at 2 dpi with RNeasy mini-kit [Qiagen]. RNA was incubated with deoxyribonuclease I, amplification grade [Invitrogen] to remove residual genomic DNA. Reverse transcription was performed with SuperScript One-Step RT-PCR with Platinum *Taq* kit [Invitrogen], and the reaction products were analyzed on 1.5% agarose gel. The following primer pairs were used; GAPDH_77-96F [5′-GAG TCA ACG GAT TTG GTC GT-3′]

and GAPDH_295-314R [5′-TTG ATT TTG GAG GGA TCT CG-3′], E3L_396-415F [5′-CGG AGC TGT ACA CCA TAG CA-3′] and E3L_540–559R [5′-TAT TGA CGA GCG TTC TGA CG-3′], B18R_384-403F [5′-TGT GGT TAA ATG GGA AAG GC-3′] and B18R_1055-1074R [5′GTA CAA GGT CGC CTC GGT AA], K3L_145-168F [5′-TGC CAA GAT AGC TTC AAA GTG AGG-3′] and K3L_234-253R [5′-TTA TTC GTT GCC CAA TGC GG-3′], B8R_405-424F [5′-CAA ACG CGG TGA CAT GTG TG-3′] and B8R_560-579R [5′-TTC TGT GGC TCC CTG TGC TG-3′], IFNA_28-47F [5′-ACC CAT CTC AGC AAG CCC AG-3′] and IFNA_550-569R [5′-TGA CAA CCT CCC AGG CAC AA-3′], IFNB_202-221F [5′-TGG CAA TTG AAT GGG AGG CT-3′] and IFNB_473–493R [5′-TGC TCA TGA GTT TTC CCC TGG-3′]. The expected product sizes were as follows, GAPDH [237 bp], E3L [163 bp], B18 R [690 bp], K3L [108 bp], B8R [174 bp], IFN-α [453 bp] and IFN-β [291 bp]. The gel photographs were scanned and further analyzed for density comparison by using IQ Mac v1.2 software [Molecular Dynamics]. The density ratio of the RT-PCR products from total RNA obtained from cells transfected with *E3L*-specific siRNAs was determined with reference to the similar sample from cells infected with Vaccinia virus.

Statistical analysis

Statistically significant differences between control and test groups were determined by Student's *t* test. *P* values of ≤0.05 were considered significant. All the analyses were performed in Microsoft Excel.

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